Applications in Plant Sciences 2014 2(6): 1400021

Primer Note

Novel microsatellite markers for the high-alpine *Geum reptans* (Rosaceae)[1]

Elena Hamann[2], Halil Kesselring[2], Jürg Stöcklin[2], and Georg F. J. Armbruster[2,3]

[2]Department of Environmental Sciences, Population Biology of Plants, University of Basel, Schönbeinstrasse 6, CH-4056 Basel, Switzerland

- *Premise of the study:* *Geum reptans* reproduces by outcrossing or by the formation of stolons. Sexual and clonal reproduction are not exclusive and occur mostly simultaneously. We developed novel microsatellite primers for this species, which will be used in a study about local adaptation, phenotypic plasticity, and random molecular divergence of alpine plants.
- *Methods and Results:* Twelve microsatellite primer sets were developed for *G. reptans*, of which nine were polymorphic. Initially, the forward primers had an M13 tail, and the allelic signals of each locus were amplified using a single fluorescent-labeled M13 forward sequence. In the running phase, a multiplex PCR assay was developed using different fluorophore-labeled forward primers. Two to 11 alleles were found per locus, depending on the studied population.
- *Conclusions:* Identical multilocus genotypes (i.e., clonal offspring) were not found because individuals in our sampling were at least 4 m distant from each other. F_{ST}–Q_{ST} analysis will be applied to detect selection processes in populations of *G. reptans* across the Alps.

Key words: alpine scree; clonal reproduction; ECO500 size marker; *Geum reptans*; multiplex PCR; Rosaceae.

It is generally assumed that alpine plants are locally adapted due to strong selection in habitats characterized by severe climatic conditions and high environmental heterogeneity (Körner, 2003). However, this assumption has rarely been tested. Our research uses reciprocal transplantation experiments (RTE) and sibling analyses to estimate the degree of phenotypic plasticity vs. the degree of local adaptation in populations of several alpine plant species (Via, 1984; Kawecki and Ebert, 2004). Because each transplantation site of an RTE is also a common garden, phenotypic differentiation of plant functional traits can be compared to neutral genetic differentiation based on microsatellite data (i.e., F_{ST}–Q_{ST} analysis). This allows the deduction of the strength and direction of past selection on the measured traits and, therefore, allows testing for local adaptation (Spitze, 1993; Scheepens et al., 2013).

One of our focal alpine plants is *Geum reptans* L. (Rosaceae, Rosidae). *Geum reptans* is a diploid, perennial rosette plant usually found in high-alpine scree fields and in glacier forelands. The plant uses sexual reproduction to produce viable seeds, and also expands through vegetative aboveground stolons (Fig. 1; Weppler et al., 2006). Selfing provides no viable seeds in *G. reptans*, probably due to gametophytic self-incompatibility as in other species of the Rosaceae (see Rusterholz et al., 1993). Cross-amplification of microsatellite primers developed for the lowland rosid *G. urbanum* L. has been reported in seeds of *G. reptans* (Arens et al., 2004); however, trans-species amplification was not successful in our laboratory despite our use of the PCR protocol described by Arens et al. (2004). Hence, we have developed new polymorphic microsatellite loci in *G. reptans* to analyze neutral genetic differentiation within our study populations.

[1]Manuscript received 10 February 2014; revision accepted 26 February 2014.

The authors thank Ecogenics GmbH (Zurich-Schlieren, Switzerland) for technical support. This project was funded by a Swiss National Science Foundation grant to J.S. (no. 31003A_135611/1).

[3]Author for correspondence: g.armbruster@unibas.ch

doi:10.3732/apps.1400021

METHODS AND RESULTS

We sampled three geographically distinct populations from the Swiss Alps (Davos: 46°44′47.46″N, 9°56′47.70″E; Furka: 46°33′24.28″N, 8°24′45.40″E; Zermatt: 45°59′16.69″N, 7°40′40.73″E). Tissue samples from young leaves of 20 randomly selected individuals were collected from each population in summer 2012. Sampled individuals were separated by at least 4 m to minimize the risk of resampling identical clones (Pluess and Stöcklin, 2004, p. 2014). Leaf material was stored in silica in the collection of the University of Basel, section of Population Biology of Plants. Microsatellite marker development was outsourced to Ecogenics GmbH (Zurich-Schlieren, Switzerland). The genome screening technique of Ecogenics has been described previously (Kesselring et al., 2013). The total of 26,603 reads had an average length of 178 bp, and 2222 of these reads contained a microsatellite insert with a tetra- or trinucleotide of at least six repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible in 309 reads.

To find allelic polymorphisms, a test sample of $N = 15$ individuals was used. For the screening of loci for polymorphism and PCR functionality, a PCR strategy (see details in Kesselring et al., 2013) that involved M13-tailing at the 5′-end of each forward primer was used (Schuelke, 2000). Finally, nine out of 12 loci provided sufficient allelic polymorphisms and robust PCR characteristics (Table 1). To facilitate subsequent genotyping, the PCR of the nine loci were combined in three multiplex assays using fluorophore labeling (Table 1). For each locus, a fraction of the forward primer was labeled with a fluorophore and complemented with nonlabeled forward primer and reverse primer to a concentration of 10 μM primer master mix (see Table 1, footnote 1). Subsequently, 20 individuals from each of the three populations (Davos, Furka, Zermatt) were tested (a total of $N = 60$ individuals, see Table 2). PCR was done in a final volume of 10 μL, and contained 1 μL PCR stock buffer (QIAGEN, Hilden, Germany) with 15 mM $MgCl_2$ and 200 μM dNTPs, 0.3 μL primer master mix

Fig. 1. Reproducing individual of *Geum reptans* in a glacier forefield. The stolons (red arrows) root at the end, eventually forming a new clonal plant next to its 'mother.' This reproductive mode with sexual flowers and vegetative, aboveground stolons is comparable to strawberries (e.g., *Fragaria vesca* L.).

of each of the three loci, 0.5 unit HotStar Taq polymerase (QIAGEN), and 2–10 ng DNA. Cycling conditions were: denaturation at 95°C for 15 min, followed by start PCR at 94°C for 30 s, 56°C for 90 s, and 72°C for 60 s in 35 cycles. Final elongation was set to 72°C for 30 min. After PCR, samples were mixed with ECO500 size standard (provided by Ecogenics) and loaded on an ABI3730 sequencer (Applied Biosystems, Carlsbad, California, USA). This size marker is suitable for accurate sizing in the range of 50–500 bp. ECO500 was labeled with orange DY-630 dye (Dyomics GmbH, Jena, Germany) and comprised the following base pair fragments: 75, 102, 124, 148, 171, 207, 229, 260, 274, 311, 321, 349, 374, 395, 419, 455, 473, and 497 bp. Allelic assignment of the electropherograms was done with GeneMarker version 1.80 (SoftGenetics, State College, Pennsylvania, USA). Data were cross-checked for repeatability. Eight of the 60 individuals were retested, starting again with DNA extraction of the silica gel–dried leaves and microsatellite fingerprinting. Hence, 72 fingerprints (i.e., 8 samples · 9 loci) of the first run were opposed to 72 fingerprints of the repetition run. Just one pairwise comparison differed, which is an error rate of 1.4%. The error occurred due to inconsistent allelic assignment of an individual at locus 015615. Instead of being heterozygous, the individual was interpreted in the repetition run as homozygous. Two loci showed background noise (loci 015967 and 013998; Table 1), i.e., we interpreted the constant occurrence of an additional peak as a mismatch. Moreover, at locus 013998 an allele of 151 bp was found to occur at a frequency of 5%. This allele was binned with the common allele of 150 bp because of potential stuttering (see Table 1). The same was done with the 144-bp allele (frequency of 8%; binning with 143 bp) at locus 002235 (Table 1). The polished data set was written in GENEPOP format (Rousset, 2008). Three software packages were used: GENEPOP on the Web for general index calculations and tests on linkage disequilibrium (Rousset, 2008; http://genepop.curtin.edu.au/), MICRO-CHECKER (van Oosterhout et al., 2004) for tests on potential null alleles with a prior value of maximum allele length of 250 bp and a 95% confidence limit, and GenAlEx version 6.2 (Smouse et al., 2008; Beck et al., 2008) for finding identical multilocus clones.

Two to 11 alleles were found per locus, depending on the studied population (Table 2). Observed (H_o) and expected heterozygosities (H_e) were in good agreement overall, indicating sexual outcrossing and random mating of alleles. However, three loci showed obvious deviation between H_o and H_e (Table 2). The presence of null alleles was suggested by MICRO-CHECKER for loci 002235, 007389, and 011721 in some populations (Table 2). The independent evolution of the microsatellite loci was tested with linkage analysis. There was no linkage disequilibrium among pairs of loci across all populations (all $P >$ 0.09). In a further step, we searched for identical multilocus genotypes because of clonal reproduction by stolons. Six of the 60 individuals had to be excluded from the analysis because they had missing values at some loci (000-allele code in GENEPOP). We did not find identical multilocus genotypes in the 54 remaining individuals, although the establishment of clonal offspring by stolons of *G. reptans* is common and was estimated to range between 53% and 74% (Weppler et al., 2006).

CONCLUSIONS

The new microsatellite markers described here proved to be valuable tools to perform population and landscape genetics studies in the clonal plant *G. reptans*, for parental analysis or further investigations of its breeding system. Observed and expected heterozygosities were in good agreement, indicating random mating of alleles and sexual outcrossing; however, null alleles might occur at some loci. Given the absence of identical multilocus genotypes, we assume that our sampling design was successful in avoiding clonal individuals and indicate that clonal offspring of *G. reptans* establish only right next to their 'mother' plants. In the near future, we will examine microsatellite data of *G. reptans* to identify neutral genetic differentiation across the Alps. Contrasting molecular differentiation with differentiation in fitness-related phenotypic traits of reciprocally transplanted populations (F_{ST}–Q_{ST} analysis) should allow detection of selection and local adaptation.

LITERATURE CITED

Arens, P., W. Durka, J. H. Wernke-Lenting, and M. J. M. Smulders. 2004. Isolation and characterization of microsatellite loci in *Geum urbanum* (Rosaceae) and their transferability within the genus *Geum. Molecular Ecology Notes* 4: 209–212.

Beck, N. R., R. Peakall, and R. Heinsohn. 2008. Social constraint and an absence of sex-biased dispersal drive fine-scale genetic structure in white-winged choughs. *Molecular Ecology* 17: 4346–4358.

Kawecki, T. J., and D. Ebert. 2004. Conceptual issues in local adaptation. *Ecology Letters* 7: 1225–1241.

Table 1. Characteristics of the newly developed microsatellite markers for *Geum reptans*.

Locus[1]	GenBank accession no.	Primer sequence (5′–3′)	Repeat motif	Amplicon length (bp)	Comment
015967 (a)	KJ439055	F: ACGGGTCTCTCTTCACTTGG R: TGACCATACTCATTCGCCCC	$(TG)_{13}$	125–145	Depending on genotype, mismatch signal between 122 and 126 bp.
011721 (a)	KJ439056	F: AAAACCCTAGCCTTCGTCGC R: ATGTTAAGTGCAGCGGTTCG	$(TC)_{11}$	92–121	
013998 (a)	KJ439057	F: GAGCCACACTGAAAGCCATC R: GCCACTCTCAGTATCTTCCTCC	$(AC)_{11}$	125–150	Depending on genotype, mismatch signal between 124 and 125 bp. The 151-bp allele was binned with the common allele of 150 bp.
002235 (b)	KJ439058	F: TCCGGTCCACCAAAGGATAG R: CTTGCCTTTTCCATGGGCTC	$(CT)_{12}$	143–171	The 144-bp allele was binned with the common allele of 143 bp.
003651 (b)	KJ439059	F: CCACCTACAGTACGGACGAC R: ACCCCAATTCATTCGACACG	$(GA)_{12}$	125–221	
011534 (b)	KJ439060	F: CGCCCAAAATCAATCCATCAC R: GTACACCTTTGCTCCCCCTC	$(AG)_{14}$	95–189	
015615 (c)	KJ439061	F: TTTTGGATTGGACTACATAGACAG R: CAGTACCTGGAATCTGGGGG	$(CA)_{12}$	137–160	
013198 (c)	KJ439062	F: TGTGATCGATTAACTGCTGACG R: CACTCCCTCCAGCTCAGTTC	$(AG)_{11}$	131–182	
007389 (c)	KJ439063	F: AGTAGACCGGCCTTGATTCC R: ACCACAAAAGTAATGAAGCTTTTCG	$(TC)_{12}$	120–142	
07238[2]	KJ439064	F: ACAAAAATGGCGAGAGCATC R: CTTTGGTACGGCCCATTTCG	$(TGA)_7$	180–186	Only 8 of the 15 test individuals gave readable amplicons; three alleles with 180 bp, 183 bp, and 186 bp were found.
14769[2]	KJ439065	F: TGTGTGTGTTTTGCCCTAGC R: AAAGTACCCCATCCCAGCTC	$(TC)_{11}$	94	monomorph; 1 allele with 94 bp
26238[2]	KJ439066	F: CGTCGCTCTCTCTATCTACCC R: GAGAGTGAGGTTTTCCGGC	$(CCG)_7$	80	monomorph; 1 allele with 80 bp

[1]Three multiplex PCR assays (a, b, c) were performed. The following fluorescent dyes were used: ATTO532 for the forward primer at locus 011721; ATTO565 for the forward primers at loci 013998, 011534, and 007389; FAM for the forward primers at loci 015967, 002235, and 015615; ATTO550 for the forward primers at loci 003651 and 013198. In the multiplex PCR assays, the ratio of fluorophore-labeled forward primer and unlabeled forward primer was between 0.11 and 0.52 (see text). For example, the primer master mix of locus 011721 contained 0.5 μL ATTO532-labeled forward primer, 4.5 μL unlabeled forward primer, 5 μL reverse primer, and 40 μL ddH_2O, in a final concentration of 10 μM total forward primer and 10 μM total reverse primer. Of this master mix, 0.3 μL were pipetted into the PCR tube together with two other primer master mix solutions (see text).

[2]Three of the tested loci were excluded. These loci were either monomorphic in the test sample of N = 15 individuals or could be amplified only in a subset of the 15 test individuals.

Kesselring, H., E. Hamann, J. Stöcklin, and G. F. J. Armbruster. 2013. New microsatellite markers for *Anthyllis vulneraria* (Fabaceae), analyzed with Spreadex gel electrophoresis. *Applications in Plant Sciences* 1: 1300054.

Körner, C. 2003. Alpine plant life: Functional plant ecology of high mountain ecosystems, 2nd ed. Springer, Heidelberg, Germany.

Pluess, A., and J. Stöcklin. 2004. Population genetic diversity of the clonal plant *Geum reptans* (Rosaceae) in the Swiss Alps. *American Journal of Botany* 91: 2013–2021.

Rousset, F. 2008. GENEPOP'007: A complete reimplementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

Rusterholz, H.-P., J. Stöcklin, and B. Schmid. 1993. Populationsbiologische Studien an *Geum reptans* L. *Verhandlungen der Gesellschaft für Ökologie* 22: 337–346.

Scheepens, J. F., E. S. Frei, and J. Stöcklin. 2013. Glacial history affected phenotypic differentiation in the alpine plant, *Campanula thyrsoides. PLoS ONE* 8: e73854.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. A poor man's approach to genotyping for research and high-throughput diagnostics. *Nature Biotechnology* 18: 233–234.

Smouse, P. E., R. Peakall, and E. Gonzales. 2008. A heterogeneity test for fine-scale genetic structure. *Molecular Ecology* 17: 3389–3400.

Table 2. Results of initial primer screening of nine polymorphic microsatellite loci in three populations of *Geum reptans* (each with N = 20 individuals).

	Davos, Switzerland			Furka, Switzerland			Zermatt, Switzerland		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
015967	4	0.6	0.57	4	0.75	0.65	2	0.3	0.39
011721	6	0.45	0.66*	8	0.70	0.79	7	0.45	0.73*
013998	6	0.60	0.56	3	0.75	0.63	2	0.05	0.14
002235	5	0.50	0.71*	3	0.35	0.59*	3	0.10	0.19
003651	11	0.70	0.77	11	0.90	0.83	4	0.65	0.65
011534	8	0.70	0.76	8	0.80	0.86	9	0.85	0.83
015615	9	0.65	0.81	10	0.90	0.86	6	0.60	0.67
013198	9	0.75	0.81	9	0.85	0.85	10	0.90	0.82
007389	9	0.25	0.86*	5	0.15	0.77*	4	0.60	0.53

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

*Indicates potential null alleles (see text).

Spitze, K. 1993. Population-structure in *Daphnia obtusa*: Quantitative genetic and allozyme variation. *Genetics* 135: 367–374.

van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.

Via, S. 1984. The quantitative genetics of polyphagy in an insect herbivore. II. Genetic correlations in larval performance within and among host plants. *Evolution* 38: 896–905.

Weppler, T., P. Stoll, and J. Stöcklin. 2006. The relative importance of sexual and clonal reproduction for population growth in the long-lived alpine plant *Geum reptans*. *Journal of Ecology* 94: 869–879.